Description

Alkaline Protease

FIELD OF THE INVENTION

The present invention relates to an alkaline protease and to a gene encoding the same.

BACKGROUND OF THE INVENTION

Protease has long been used in industry, and has found utility in a diversity of fields, including detergents such as laundry detergents, fiber modifying agents, leather processing agents, cosmetic compositions, bath additives, food-modifying agents, and pharmaceuticals. Of these, proteases for detergent use are produced in the largest amounts on an industrial scale. Examples of such known proteases include Alcalase, Savinase (registered trademarks; Novozymes), Maxacal (registered trademark; Genencor), Blap (registered trademark; Henkel), and KAP (Kao Corporation).

The purpose of incorporating protease into a detergent is to degrade protein soil adhering to clothes. Such soil actually is a "complex" soil formed of a plurality of organic and inorganic components, including not only proteins but also lipids originating from sebum, solid particles, and other substances. Therefore, demand has arisen for a detergent having excellent detergency against such complex soil.

Under the above situation, some of the present inventors have previously discovered several species of alkaline protease which have a molecular weight of about 43,000, exhibit a sufficient casein-degrading activity even in the presence of a fatty acid at a high concentration, and also exhibit excellent detergency not only to proteins but also to complex soils which include sebum and other substances, and filed a patent application therefor (see Patent Publication WO99/18218). Since the discovered alkaline proteases differ from subtilisin (which is a conventionally known serine protease derived from a microorganism belonging to the genus *Bacillus*) in terms of molecular weight, primary structure, enzymological characteristics and resistance to oxidants (the alkaline proteases are strongly resistant to oxidants) their classification into a new subtilisin subfamily has been proposed (Saeki *et al.*, Biochem. Biophys. Res. Commun., 279, 313-319, 2000).

Incorporating such proteases into a detergent requires several steps, including concentration of a culture, drying, and granulation. Also, deactivation of proteases, which may otherwise occur during storage of the detergent, must be prevented.

In addition, researchers have recognized the problem that when a gene coding for such a protease is modified to give mutants having an enhanced specific activity and high production, some of such mutants show thermal stability lower

than that of a protease before undergoing mutation. Thus, there has arisen a demand to improve thermal stability of the enzymes to solve the mentioned problems.

Accordingly, the present invention provides an alkaline protease which has excellent detergency against complex soil as well as enhanced thermal stability.

SUMMARY OF THE INVENTION

The present invention provides an alkaline protease having an amino acid sequence wherein one or more amino acid residues selected from those located at (a) position 63, (b) position 89, (c) position 120, (d) positions 63 and 187, (e) position 226, (f) position 296, (g) position 304 of the amino acid sequence of SEQ ID NO: 1, or at positions corresponding to these positions are the following amino acid residues, respectively:

Position (a): serine,

Position (b): histidine,

Position (c): arginine

Position (d): serine,

Position (e): tyrosine,

Position (f): valine, and

Position (g): serine.

The present invention also provides a gene encoding the alkaline protease.

The present invention also provides a vector comprising the gene, and a transformant containing the vector.

The present invention also provides a detergent composition containing the above-described alkaline protease.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows amino acid sequence alignment of protease having 80% or higher homology with the amino acid sequence of SEQ ID NO: 1.

FIG. 2 shows improvement in thermal stability of the alkaline protease of an embodiment of the present invention after treatment at 70°C for 10 minutes in a borate buffer (pH 10, 50 mM).

FIG. 3 shows improvement in thermal stability of the alkaline protease of an embodiment of the present invention after treatment at 80°C for 10 minutes in 2mM calcium chloride.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have searched for a new enzyme which is endowed with the characteristics of the aforementioned alkaline protease and also with for example, improved thermal stability, and have found that such an enzyme, which is a certain alkaline protease, requires the presence of specified amino acid residue(s) at specified position(s) of the amino acid sequence of the alkaline protease.

The alkaline protease of the present invention has an amino acid sequence wherein one or more amino acid residues

selected from those located at (a) position 63, (b) position 89, (c) position 120, (d) positions 63 and 187, (e) position 226, (f) position 296, (g) position 304 of the amino acid sequence of SEQ ID NO: 1, or at positions corresponding to these positions are the following amino acid residues, respectively:
position (a): serine, position (b): histidine, position (c): arginine, position (d): serine,
position (e): tyrosine, position (f): valine, and position(g): serine.

Namely, the alkaline protease of the present invention is a protease that has been engineered such that one or more amino acid residues selected from the above-mentioned positions (a) to (g) of an alkaline protease having an amino acid sequence of SEQ ID NO: 1, or amino acid residue(s) of another alkaline protease at position(s) corresponding to the above-mentioned positions (a) to (g), are specified amino acid residue(s), and may be of a wild type, mutant(s) of the wild type, or mutant(s) created by artificial mutagenesis.

As used herein, "another alkaline protease" may be either a wild type enzyme or a mutant of the wild type enzyme. Preferably, "another alkaline protease" exhibits resistance to oxidants and has a molecular weight of 43,000 ± 2,000 as determined by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis), and as an example thereof, mention may be given of an alkaline protease having such an amino acid sequence that exhibits 80% or higher homology with the amino

acid sequence of SEQ ID NO: 1. More preferably, "another alkaline protease" is an enzyme which has an amino acid sequence that exhibits 80% or higher homology with the amino acid sequence of SEQ ID NO: 1; acts in an alkaline region of pH 8 or higher; exhibits resistance to oxidants; shows 80% or higher residual activity after treatment at 50°C for 10 minutes at pH 10; is inhibited by diisopropylfluorophosphate (DFP) and phenylmethanesulfonyl fluoride (PMSF); and has a molecular weight of 43,000 ± 2,000 as determined by SDS-PAGE. As used herein, the expression "exhibit resistance to oxidants" means that after an alkaline protease is left to stand at 30°C for 20 minutes in 20 mM Britton-Robinson buffer (pH 10) containing 50 mM hydrogen peroxide and 5 mM calcium chloride, the alkaline protease maintains a residual activity of at least 50%.

Examples of the "alkaline protease having an amino acid sequence of SEQ ID NO: 1" include KP43 [derived from *Bacillus* sp. KSM-KP43 (FERM BP-6532), Patent Publication WO99/18218]. Examples of the "alkaline protease having an amino acid sequence that exhibits 80% or higher homology with the amino acid sequence of SEQ ID NO: 1" include protease KP9860 (GenBank Accession No. AB046403) [derived from *Bacillus* sp. KSM-KP9860 (FERM BP-6534), International Patent Publication WO99/18218]; protease 9865 (GenBank Accession No. AB084155) [derived from *Bacillus* sp. KSM-9865 (FERM P-1592), Japanese Patent Application Laid-Open (*kokai*) No. 2003-199559]; protease E-1 (GenBank Accession No. AB046402) [derived from

Bacillus No. D-6 (FERM P-1592), Japanese Patent Application Laid-Open (*kokai*) No. 49-71191]; protease Ya (GenBank Accession No. AB046404) [derived from *Bacillus* sp. Y (FERM BP-1029), Japanese Patent Application Laid-Open (*kokai*) No. 61-280268]; protease SD521 (GenBank Accession No. AB046405) [derived from *Bacillus* SD521 (FERM P-11162), Japanese Patent Application Laid-Open (*kokai*) No. 3-191781]; protease A-1 (GenBank Accession No. AB046406) [derived from NCIB12289, Patent Publication WO88/01293]; protease A-2 [derived from NCIB12513, Patent Publication WO98/56927]; mutant proteases described in Japanese Patent Application Laid-Open (*kokai*) Nos. 2002-218989 and 2002-306176; mutants obtained through substitution of position 251 of the amino acid sequence of SEQ ID NO: 1 by asparagine, threonine, isoleucine, valine, leucine or glutamine; mutants obtained through substitution of position 256 of the same amino acid sequence by serine, glutamine, asparagine, valine, or alanine (Japanese Patent Application Laid-Open *(kokai)* No. 2003-125783); a mutant obtained through substitution of position 65 of the amino acid sequence of SEQ ID NO: 1 by proline; a mutant obtained through substitution of position 101 of the same amino acid sequence by asparagine; mutants obtained through substitution of position 273 of the same amino acid sequence by isoleucine, glycine, or threonine; mutants obtained through substitution of position 320 of the same amino acid sequence by phenylalanine, valine, threonine, leucine, isoleucine, or glycine; mutants obtained through substitution of position

359 of the same amino acid sequence by serine, leucine, valine, isoleucine, or glutamine, mutants obtained through substitution of position 387 of the same amino acid sequence by alanine, lysine, glutamine, glutamic acid, arginine, or histidine (Japanese Patent Application Laid-Open *(kokai)* No. 2004-000122); mutants obtained through substitution of position 163 of the amino acid sequence of SEQ ID NO: 1 by histidine, aspartic acid, phenylalanine, lysine, asparagine, serine, isoleucine, leucine, glutamine, threonine or valine; mutants obtained through substitution of position 170 of the same amino acid sequence by valine or leucine; mutants obtained through substitution of position 171 of the same amino acid sequence by alanine, glutamic acid, glycine, or threonine (Japanese Patent Application Laid-Open *(kokai)* No. 2004-057195); and an alkaline protease having an amino acid sequence that exhibits a 80% or higher, preferably 87% or more, more preferably 90% or more, still more preferably 95% or more, homology with any of the above listed amino acid sequences.

Homology of amino acid sequences can be preferably determined by the Lipman-Pearson method (Science, 227, 1435, 1985).

"Amino acid residues located at positions corresponding to the positions ……" can be identified by comparing amino acid sequences of alkaline proteases by means of a known algorithm such as the Lipman-Pearson method, to thereby assign maximum homology to conserved amino acid residues

present in the amino acid sequences. When the amino acid sequences of proteases are aligned by means of such method, regardless of insertion or deletion occurred in the amino acid sequences, the positions of the homologous amino acid residues can be determined in each of the proteases. Conceivably, homologous amino acid residues are located at the same positions in the three-dimensional structure of protease, whereby analogous effects are obtained in terms of specific functions of the intended protease.

As shown in FIG. 1, in which amino acid sequences are aligned by means of the aforementioned method, the amino acid residue at "(a) position 63 of the amino acid sequence of SEQ ID NO: 1" is asparagine. Through use of the method described in the above paragraph, an amino acid residue at a position corresponding to that position can be identified as, for example, asparagine at position 63 in case of protease KP9860. In this connection, the amino acid residue at that position is preferably serine.

The amino acid residue at "(b) position 89 of the amino acid sequence of SEQ ID NO: 1" is glutamine. Through use of the above-described method, an amino acid residue at a position corresponding to that position can be identified as, for example, glutamine at position 88 in case of protease E-1. Preferably, the amino acid residue at that position is histidine.

The amino acid residue at "(c) position 120 of the amino acid sequence of SEQ ID NO: 1" is serine. Through use

of the above-described method, an amino acid residue at a position corresponding to that position can be identified as, for example, serine at position 119 in case of protease A-2. Preferably, the amino acid residue at that position is arginine.

The amino acid residues at "(d) positions 63 and 187 of the amino acid sequence of SEQ ID NO: 1" are both asparagine. Through use of the above-described method, amino acid residues at positions corresponding to those positions can be identified as, for example, asparagines at positions 63 and 186 in case of protease SD-521. Preferably, both of the amino acid residues at those positions are serine.

The amino acid residue at "(e) position 226 of the amino acid sequence of SEQ ID NO: 1" is phenylalanine. Through use of the above-described method, an amino acid residue at a position corresponding to that position can be identified as, for example, phenylalanine at position 225 in case of protease Ya. Preferably, the amino acid residue at that position is tyrosine.

The amino acid residue at "(f) position 296 of the amino acid sequence of SEQ ID NO: 1" is isoleucine. Through use of the above-described method, an amino acid residue at a position corresponding to that position can be identified as, for example, isoleucine at position 296 in case of protease 9865. Preferably, the amino acid residue at that position is valine.

The amino acid residue at "(g) position 304 of the

amino acid sequence of SEQ ID NO: 1" is asparagine. Through use of the above-described method, an amino acid residue at a position corresponding to that position can be identified as, for example, aspartic acid at position 303 in case of protease E-1. Preferably, the amino acid residue at that position is serine.

Specific examples of the positions and amino acid residues corresponding to (a) position 63, (b) position 89, (c) position 120, (d) positions 63 and 187, (e) position 226, (f) position 296, and (g) position 304 of the amino acid sequence (SEQ ID NO: 1) of protease KP43, are shown below by way of some preferred examples of the aforementioned "another alkaline protease" (Table 1).

Table 1

Position	Protease							
	KP43	KP9860	9865	E-1	Ya	SD-521	A-1	A-2
(a)	Asn63	Asn63	Asn63	Asn63	Ser63	Asn63	Asn63	Asn63
(b)	Gln89	Gln89	Gln89	Gln88	Gln88	Gln88	Gln89	Gln88
(c)	Ser120	Ser120	Ser120	Asn119	Asn119	Asn119	Ser120	Ser119
(d)	Asn63 Asn187	Asn63 Asn187	Asn63 Asn187	Asn63 Asn186	Ser63 Asn186	Asn63 Asn186	Asn63 Asn187	Asn63 Asn186
(e)	Phe226	Tyr226	Phe226	Phe225	Phe225	Phe225	Phe226	Tyr225
(f)	Ile296	Val296	Ile296	Val295	Val295	Val295	Ile296	Val295
(g)	Asn304	Asn304	Asn304	Asp303	Asp303	Asp303	Asn304	Asn303

Among the positions (a) to (g) of the amino acid residues of the alkaline protease of the present invention, two or more positions may be concurrently selected, so long as enzyme activity and enzyme characteristics remain unchanged. Preferred examples of two or more positions being selected concurrently are shown below. Amino acids are designated by the three letter codes, and the symbol "+"

means an additional substitution.

Specific examples of double substitution of amino acid residues include Asn63Ser + Asn187Ser, Asn63Ser + Ile296Val, Asn187Ser + Ile296Val, and Ser120Arg + Phe226Tyr, wherein Asn63Ser + Asn187Ser is particularly preferred. Combinations of three or more substitutions may also be employed.

When the alkaline protease of the present invention is a mutant, the alkaline protease before undergoing mutagenesis (which may be referred to as "parent alkaline protease") is either a "protease having an amino acid sequence of SEQ ID NO: 1" or the aforementioned "another alkaline protease." When the parent alkaline protease is subjected to mutation at a predetermined site thereof, the alkaline protease of the present invention can be obtained. For example, when an amino acid residue at a position selected from among the aforementioned positions (a) to (g) of the amino acid sequence of SEQ ID NO: 1 of protease KP43 (or an amino acid residue at a position corresponding to any of the above positions in the amino acid sequence of another alkaline protease) is replaced by another amino acid residue, the alkaline protease of the present invention can be obtained.

The alkaline protease of the present invention may be obtained through, for example, the following steps. Briefly, a cloned gene encoding parent alkaline protease (SEQ ID NO: 2; a gene encoding SEQ ID NO: 1, or a mature enzyme region, is represented by the sequence starting from the 619th codon) is mutated, and by use of the thus-mutated gene an

appropriate host bacterium is transformed, followed by culturing of the recombinant host bacterium and collecting the alkaline protease product of the invention from the culture. Cloning of the gene encoding the parent alkaline protease may be carried out through a generally employed gene recombination technique. For example, a method described in Patent Publication WO99/18218 or Patent Publication WO98/56927 may be employed.

Means for carrying out mutagenesis of the gene encoding the parent alkaline protease may be random mutagenesis or site-directed mutagenesis which is commonly performed. More specifically, mutagenesis of the gene may be carried out by use of, for example, a Site-Directed Mutagenesis System Mutan-Super Express Km kit (Takara). Alternatively, by means of recombinant PCR (polymerase chain reaction; see "PCR Protocols," Academic Press, New York, 1990), an arbitrary sequence of the gene can be replaced by the arbitrary sequence of another gene.

Production of the protease of the present invention by use of the thus-obtained mutant gene may be carried out, for example, by ligating the mutated gene to a DNA vector capable of stably amplifying the gene, to thereby transform host bacteria. Alternatively, the mutant gene may be introduced into chromosomal DNA of a host bacterium capable of stably maintaining the gene. Examples of the host bacterium which satisfies these requirements include bacteria belonging to the genus *Bacillus*, *Escherichia coli*, mold, yeast, and

actinomycetes. Any of these microorganisms is inoculated into a culture medium containing an assimilable carbon source, nitrogen source, and other essential nutrients, and culturing is carried out according to a customary method.

From the thus-obtained culture, alkaline protease may be collected and purified by means of customary methods for collecting and purifying enzymes. For example, the culture is subjected to centrifugation or filtration to thereby remove cells, and the enzyme of interest is obtained from the culture supernatant by means of a routine purification technique. The thus-obtained enzyme solution may be employed as is. Alternatively, the enzyme solution may further be subjected to purification, crystallization, powdering, or granulation, any of which may be carried out according to a known method.

The thus-produced alkaline protease of the present invention exhibits oxidant resistance and maintains casein-degrading activity even in the presence of a fatty acid at a high concentration. The alkaline protease has a molecular weight of 43,000 ± 2,000 as determined by SDS-PAGE, and is active within the alkaline region. Moreover, the alkaline protease exhibits a newly acquired property; i.e., improved thermal stability compared with that of the parent alkaline protease.

Thus, the alkaline protease of the present invention is useful as an enzyme to be incorporated in a variety of detergent compositions.

No particular limitation is imposed on the amount of the protease of the present invention to be incorporated into a detergent composition, so long as the alkaline protease exhibits activity. The preferred amount is 0.1 to 5,000 PU per kg of detergent composition, more preferably 500 PU or less, in consideration of cost and other factors.

The detergent composition of the present invention may further contain a variety of enzymes in addition to the protease of the present invention. Examples of such additional enzymes include hydrolase, oxidase, reductase, transferase, lyase, isomerase, ligase, and synthetase. Of these, preferred enzymes include proteases other than those of the present invention, cellulase, keratinase, esterase, cutinase, amylase, lipase, pullulanase, pectinase, mannanase, glucosidase, glucanase, cholesteroloxidase, peroxidase, and laccase, among which the proteases, cellulase, amylase, and lipase are more preferred. Examples of the proteases include commercially available ones such as Alcalase, Esperase, Savinase, Everlase, and Kannase (all are resistered trademarks; Novozymes), Properase and Purafect (resistered trademarks; Genencor); and KAP (Kao Corp.). Examples of cellulase include Celluzyme and Carezyme (resistered trademarks; Novozymes); and KAC, alkaline cellulase produced by *Bacillus* sp. KSM-S237 disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 10-313859, and mutated alkaline cellulase disclosed in Japanese Patent Application Laid-Open *(kokai)* No. 2003-313592 (these are products of Kao

Corp.). Examples of amylase include Termamyl and Duramyl (registered trademarks; Novozymes), Purastar (registered trademark; Genencor), and KAM (Kao Corp.). Examples of lipase include Lipolase and Lipolase Ultra (registered trademarks; Novozymes).

When a protease species other than the protease of the present invention is incorporated into a detergent composition together with the protease of the present invention, its amount is preferably 0.1 to 500 PU per kg of detergent composition. When cellulase is incorporated in combination, the amount of cellulase is preferably 300 to 3,000,000 KU per kg of detergent composition, based on the unit (KU) determined through the enzyme activity determination method described in paragraph [0020] of Japanese Patent Application Laid-Open (*kokai*) No. 10-313859.

When amylase is incorporated in combination, its amount is preferably 50 to 500,000 IU per kg of detergent composition based on the unit (IU) determined through the amylase activity determination method described in paragraph [0040] of Japanese Patent Application Laid-Open (*kokai*) No. 11-43690.

Moreover, when lipase is incorporated in combination, its amount is preferably 10,000 to 1,000,000 LU per kg of detergent composition based on the unit (LU) determined through the lipase activity determination method described in Example 1 of Japanese *Kohyo* (PCT) Patent Publication No. 8-500013.

Known detergent components may be incorporated into the detergent composition of the present invention. Examples of such known detergent components include the following substances.

(1) Surfactant

Generally, a surfactant is incorporated into the detergent composition in an amount of 0.5 to 60 mass%. In particular, the amount of surfactant is preferably 10 to 45 mass% for preparing a powdery detergent composition, and 20 to 50 mass% for preparing a liquid detergent composition. When the detergent composition of the present invention serves as a bleach composition or a detergent composition for an automated dishwasher, a surfactant is typically incorporated in an amount of 1 to 10 mass%, preferably 1 to 5 mass%.

Examples of the surfactant employed in the detergent composition of the present invention include an anionic surfactant, a nonionic surfactant, an amphoteric surfactant, a cationic surfactant, and a combination thereof. Of these, an anionic surfactant and a nonionic surfactant are preferred.

Examples of a preferred anionic surfactant include a sulfate ester salt of C10-C18 alcohol, a sulfate ester salt of an alkoxylated product of C8-C20 alcohol, an alkylbenzenesulfonate salt, a paraffinsulfonate salt, an α-olefinsulfonate salt, an α-sulfo fatty acid salt, and an α-sulfo fatty acid alkyl ester salt or a fatty acid salt. In

the present invention, a linear C10-C14 (preferably C12-C14) alkylbenzenesulfonic acid salt is more preferred. The counter ion is preferably an alkali metal or an amine, and sodium and/or potassium, monoethanol amine, or diethanol amine is more preferred.

Examples of a preferred nonionic surfactant include a polyoxyalkylene alkyl (C8-C20) ether, an alkylpolyglycoside, a polyoxyalkylene alkyl (C8-C20) phenyl ether, a polyoxyalkylene sorbitan fatty acid (C8-C22) ester, a polyoxyalkylene glycol fatty acid (C8-C22) ester, and a polyoxyethylene polyoxypropylene block polymer. A more preferred nonionic surfactant is a polyoxyalkylene alkyl ether [having an HLB value (as calculated through the Griffin method) of 10.5 to 15.0, preferably 11.0 to 14.5] which is obtained by adding 4 to 20 moles of alkyleneoxide (e.g., ethyleneoxide and propyleneoxide) to a C10-C18 alcohol.

(2) Divalent metal ion scavenger

A divalent metal ion scavenger is preferably incorporated into the composition in an amount of 0.01 to 50 mass%, preferably 5 to 40 mass%. Examples of the divalent metal ion scavenger to be employed in the detergent composition of the present invention include a condensed phosphate such as a tripolyphosphate, pyrophosphate, or orthophosphate; an aluminosilicate such as zeolite; a synthesized layered crystalline silicate; a nitrilotriacetate; an ethylenediamineteraacetate; a citrate; an isocitrate; and a polyacetalcarboxylate. Of these, a

crystalline aluminosilicate (synthesized zeolite) is preferred. Among A-type, X-type, and P-type zeolites, A-type zeolite is more preferred. The synthesized zeolite preferably has an average primary particle size of 0.1 to 10 μm, more preferably 0.1 to 5 μm.

(3) Alkaline agent

An alkaline agent is preferably incorporated into the composition in an amount of 0.01 to 80 mass%, preferably 1 to 40 mass%. Examples of the alkaline agent which may be incorporated into the detergent in powder form include an alkali metal carbonate such as sodium carbonate (collectively referred to as dense ash or light ash) and an amorphous alkali metal silicate such as JIS No. 1, No. 2, or No. 3. These inorganic alkaline agents are effective for the formation of the skeleton of particles during drying of the detergent, contributing to production of a detergent of relatively hard particles with excellent flowability. Examples of alkaline agents other than the above-described substances include sodium sesquicarbonate and sodium hydrogencarbonate. A phosphate such as tripolyphosphate also acts as an alkaline agent. Examples of alkaline agents to be employed in a detergent in liquid form include, in addition to the above-described alkaline agents, sodium hydroxide and mono-, di-, or tri-ethanol amine, which can also be employed as a counter ion of a surfactant.

(4) Anti-redeposition agent

An anti-redeposition agent is preferably incorporated

into the composition in an amount of 0.001 to 10 mass%, preferably 1 to 5 mass%. Examples of the anti-redeposition agent to be employed in the detergent composition of the present invention include a polyethylene glycol, a carboxylic polymer, a polyvinyl alcohol, and a polyvinyl pyrrolidone. Of these, the carboxylic polymer exerts not only an anti-redeposition effect, but also the effect of scavenging metal ions and the effect of releasing solid soil particles from the clothing into the washing liquid. The carboxylic polymer is a homopolymer or a copolymer of, for example, acrylic acid, methacrylic acid, or itaconic acid. Examples of preferred copolymers include a copolymerized product of any of the above monomers and maleic acid. The copolymer preferably has a molecular weight of some thousands to 100,000. In addition to the above carboxylic polymers, a polymer such as poly(glycidyl acid salt), a cellulose derivative such as carboxymethyl cellulose, and an aminocarboxylic polymer such as poly(aspartic acid) are also preferred, since these substances function as a metal ion scavenger, a dispersing agent, and an anti-redeposition agent.

(5) Bleaching agent

A bleaching agent such as hydrogen peroxide or a percarbonate is incorporated into the composition, preferably in an amount of 1 to 10 mass%. When such a bleaching agent is employed, tetraacetylethylenediamine (TAED) or a bleaching activator described in, for example, Japanese

Patent Application Laid-Open (*kokai*) No. 6-316700 may be incorporated into the composition in an amount of 0.01 to 10 mass%.

(6) Fluorescent agent

Examples of a fluorescent agent which may be incorporated into the detergent composition of the present invention include a biphenyl fluorescent agent (e.g., Tinopal CBS-X) and a stilbene fluorescent agent (e.g., DM-type fluorescent agent). The fluorescent agent is preferably incorporated in an amount of 0.001 to 2 mass%.

(7) Other components

The detergent composition of the present invention may contain a builder, a softener, a reducing agent (e.g., sulfite), a deformer (e.g., silicone), a perfume, or other additives, which are known in the field of laundry detergents.

The detergent composition of the present invention can be produced through a routine method by using, in combination, the protease product of the present invention obtained through the above-described method and known detergent components as listed above. The form of the detergent may be determined in accordance with its use, and examples of the form include liquid, powder, granules, paste, and solid.

The thus-obtained detergent composition of the present invention can be used as, among others, a laundry detergent, a bleaching agent, a detergent for hard surfaces, a drainpipe

detergent, a denture detergent, or a germicidal detergent for medical instruments.

Examples

The following examples further describe and demonstrate embodiments of the present invention. The examples are given solely for the purpose of illustration and are not to be construed as limitations of the present invention.

Example 1

Phe46Leu, Tyr195Gly, and Phe46Leu+Tyr195Gly, which are mutants of an alkaline protease derived from *Bacillus* sp. KSM-KP43, are all known to be very effective in improving specific activity (Japanese Patent Application Laid-Open (*kokai*) No. 2002-218989). When these specific activity-improved mutants were treated at 70°C for 15 minutes, their residual activity was found to drop to as low as 5 to 25%, as contrasted to the residual activity of the parent alkaline protease which maintained 70 to 80% of the activity before treatment. Therefore, each of these mutants was subjected to random mutagenesis of gene for producing a mutant exhibiting improved thermal stability. Briefly, a structural gene of the mutant having a size of about 2 kb was inserted into pKF18k (Takara) so as to serve as a template DNA (30 ng). PCR was performed by use of the thus-prepared template together with Taq polymerase (2.5 U), *Bca*BEST Sequencing Primer RV-M and *Bca*BEST Sequencing Primer M13-47 (both are products of Takara; 20 pmol each), dNTP (20 pmol), Takara

Taq-added reaction buffer, and appropriate amounts of manganese sulfate and dimethyl sulfoxide. In PCR, the template DNA was denatured at 94°C for one minute, followed by 30 cycles of treatment, each cycle consisting of 94°C × one minute, 55°C × two minutes, and 72°C × three minutes. The resultant PCR product was left to stand at 72°C for 10 minutes.

The PCR product was purified by use of a High Pure PCR Product Purification kit (Roche), and eluted with sterile water (100 μL). The thus-obtained DNA fragment of about 2 kb was cleaved by *Bam*HI and *Xba*I (Roche), and then mixed with pKF18k which had been treated with the same enzyme. Ligation was allowed to proceed at 16°C for 12 hours by use of a DNA Ligation kit (ver. 2; Takara). DNA was recovered through ethanol precipitation of a reaction mixture, whereby *Escherichia coli* HB101 strains were transformed. The transformants were grown on an LB agar medium containing skim milk and kanamycin.

Transformants showing resistance to kanamycin and giving translucent plaques around colonies by degrading of the skim milk were selected and inoculated into an LB culture medium containing skim milk and kanamycin, followed by shaking of the culture at 30°C for 72 hours. The activity of culture supernatant was measured through the synthetic substrate method, which will be described hereinbelow. Measurement was performed for a supernatant system which had undergone a treatment of 70°C for 15 minutes and a

supernatant system wherein no treatment was performed, whereby residual activity after heat treatment was investigated. Mutants which was were increased by about 10% to about 50% in thermal stability compared with the level shown by the parent protease were subjected to colony PCR for gene amplification, then to purification, followed by nucleotide sequencing with a DNA Sequencer (model: 377, Applied Biosystems) employing a Big Dye DNA sequencing kit (Applied Biosystems). The above process gave the following thermal-stability -improved mutants. They are a mutant in which asparagine at position 63 was replaced by serine, a mutant in which glutamine at position 89 was replaced by histidine, a mutant in which serine at position 120 was replaced by arginine, a mutant in which asparagine at position 187 was replaced by serine, a mutant in which phenylalanine at position 226 was replaced by tyrosine, a mutant in which isoleucine at position 296 was replaced by valine, a mutant in which asparagine at position 304 was replaced by serine, and a mutant in which asparagines at positions 63 and 187 were both replaced by serines.

Example 2

The points of mutation that were found to be effective for enhancing thermal stability in Example 1 were individually introduced to protease KP43 of SEQ ID NO: 1, followed by site-directed mutagenesis for evaluation of thermal stability.

A template plasmid for mutagenesis was constructed by

introducing, into *Bam*HI- and *Xba*I-cleaved sites of a pKF18K's multicloning site, a gene (SEQ ID NO: 2) coding for protease KP43.

Takara LA Taq (Takara) was employed in PCR for site-directed mutagenesis. Mutagenesis PCR was carried out by use of a selection primer whose 5'-end had been phosphorylated (20 pmol; included in a Mutan Super Express Km kit), each of primers 1 to 7 (SEQ ID NOs: 3 to 9; primers for mutagenesis; 20 pmol), and a template plasmid (30 ng). Reaction conditions of PCR were as follows. Firstly, the template DNA was denatured at 94°C for one minute, then 30 cycles of treatment, each cycle consisting of 94°C × one minute, 55°C × one minute, and 72°C × four minutes, were performed. The resultant PCR fragments were purified and used as primers. By use of the primers, a template plasmid (30 ng) and LA Taq, another PCR was carried out. Reaction conditions of this PCR were as follows. Thirty cycles of treatment, each cycle consisting of 94°C × one minute, 55°C × two minutes, and 72°C × four minutes, were performed. The resultant PCR product was purified and subjected to a ligation reaction. Subsequently, *Escherichia coli* MV1184 strains were transformed, whereby mutation-introduced plasmid was obtained. The nucleotide sequence of alkaline protease gene of the resultant plasmid was determined, and the sites of mutation were confirmed.

As representative examples of combinations of mutation, double mutants were created through PCR on the basis of the

above-prepared mutants and primers 1 to 7.

In order to produce and evaluate mutation-introduced alkaline protease, *Bacillus* sp. KSM-9865 (FERM P-18566) and pHA64 (Japanese Patent Application Laid-Open (*kokai*) No. 2000-287687: having *Bam*HI- and *Xba*I-cleaved sites downstream of promotor 64) were employed, since they were considered to serve as a suitable system for this purpose for the reason that pHA64 is capable of replicating in *Bacillus* bacteria. Each of the above-prepared mutation-introduced plasmids was treated with *Bam*HI and *Xba*I, and then mixed with pHA64 which had been treated with the same enzymes. Ligation was performed through use of a DNA Ligation kit (ver. 2; Takara). The DNA product was recovered from the ligase reaction solution through ethanol precipitation and employed in the subsequent transformation step.

The strain KSM-9865 which had undergone the transformation step were grown on a skim milk-containing alkaline agar medium [skim milk (Difco) (1% (w/v)), bactotryptone (Difco) (1%), yeast extract (Difco) (0.5%), sodium chloride (1%), agar (1.5%), sodium carbonate (0.05%), and tetracycline (15 ppm)]. Whether or not a mutated protease gene had been introduced to the strain KSM-9865 was determined on the basis of halo formation. The resultant transformants were inoculated into a seed culture medium (5 mL) [6.0% (w/v) polypeptone S, 0.05% of yeast extract, 1.0% of maltose, 0.02% of magnesium sulfate heptahydrate, 0.1% of potassium dihydrogenphosphate, 0.25% of sodium carbonate, and

30 ppm of tetracycline], followed by shaking of the culture for 16 hours at 30°C. The seed culture broth (1% (v/v)) was inoculated into a main culture medium (30 mL) [8% of polypeptone S, 0.3% of yeast extract, 10% of maltose, 0.04% of magnesium sulfate heptahydrate, 0.2% of potassium dihydrogenphosphate, 1.5% of sodium carbonate anhydrate, and 30 ppm of tetracycline], followed by shaking of the culture for three days at 30°C.

Example 3

Each of the culture supernatants was treated at 60 to 80°C for 10 minutes in 50mM borate buffer (pH 10.5: with or without 2mM calcium chloride), 50mM Tris-HCl buffer (pH 7: with 2mM calcium chloride), or a 2mM aqueous calcium chloride solution. The residual activity each of the culture supernatants was determined through the casein method, and the ratio of the residual activity to that before heat treatment was calculated. All the mutants were found to have a higher residual activity ratio as compared with the parent alkaline protease treated under the same conditions, confirming improvement in thermal stability. Some of the results are shown in FIGs. 2 and 3. These figures show the half-life period (period of time elapsed until the residual activity drops to 50% of the initial residual activity) of the proteases maintained at a certain constant temperature. As is clear from FIGs. 2 and 3, each mutant exhibited a half-value period 1.2 to 7 times that of the parent alkaline protease.

The alkaline protease mutants produced through the above process were found to exhibit enhanced thermal stability. Except for this new characteristic, they were found to maintain the characteristics of the parental alkaline protease; i.e., they exhibit oxidant resistance, maintain casein-degrading activity even in the presence of a fatty acid of high concentration, have a molecular weight of 43,000 ± 2,000 as determined by SDS-PAGE, and are active within the alkaline region.

Referential Examples

<Protease assay (synthetic substrate method)>

To a 100 mM borate buffer (pH 10.5) containing 0.05 mL of a 6 mL synthetic substrate (Glt-Ala-Ala-Pro-Leu-pNA: Peptide Institute), an enzyme solution (0.05 mL) was added, to thereby initiate reaction at 30°C for 15 minutes in a microplate reader (iEMS reader MF: LABSYSTEMS). Increase in absorbance at 414 nm was employed as an activity index. One unit of protease activity was defined as the amount of enzyme required for increasing the absorbance by 0.001 per minute under the above reaction conditions.

<Protease assay (casein method)>

A 50 mM borate buffer (pH 10.5) (1 mL) containing casein (Hammerstein method: Merck, 1% (w/v)) was maintained at 30°C for five minutes, and subsequently an enzyme solution (0.1 mL) was added to the buffer, to thereby allow reaction to proceed for 15 minutes. A reaction stopping solution (0.11M trichloroacetic acid / 0.22M sodium acetate / 0.33M

acetic acid) (2.0 mL) was added to the resultant reaction mixture, and the mixture was allowed to stand at room temperature for 30 minutes. Thereafter, the precipitation was subjected to filtration by use of a Whatman No. 1 filter, and the degradation product was quantified by means of the method described by Lowry, *et al*. Specifically, an alkaline copper solution (1% Rochelle salt: 1% copper sulfate pentahydrate: 2% sodium carbonate / 0.1N sodium hydroxide solution = 1:1:100) (2.5 mL) was added to the filtrate (0.5 mL), and the resultant mixture was allowed to stand at 30°C for 10 minutes. Subsequently, to the mixture was added a phenol reagent [obtained by diluting a commercial phenol reagent (Kanto Kagaku) two-fold with deionized water] (0.25 mL), and the resultant mixture was thoroughly stirred and left to stand at 30°C for 30 minutes. Thereafter, the absorbance of the mixture was measured at 660 nm. One unit of protease activity (1 PU) was defined as the amount of enzyme required for producing acid-soluble protein equivalent to 1 mmol of tyrosine per minute under the above reaction conditions.

Example 4

(1) Preparation of detergent

Water (465 kg) was added to a mixing bath (1 m^3) equipped with a stirring paddle. After the temperature of the water reached 55°C, a 40% (w/v) sodium polyacrylate aqueous solution (135 kg) was added to the water. The resultant mixture was stirred for 15 minutes, and then sodium

carbonate (120 kg), sodium sulfate (60 kg), sodium sulfite (9 kg), and a fluorescent dye (3 kg) were added to the mixture. The resultant mixture was further stirred for 15 minutes, zeolite (300 kg) was added to the mixture, followed by stirring for 30 minutes, to thereby yield a homogenous slurry (the water content of the slurry: 50 mass%). The slurry was sprayed through pressure spray nozzles provided in the vicinity of the top of a spray-drying tower, to thereby yield a granular base (a high-temperature gas was fed at 225°C from a lower part of the spray-drying tower, and discharged at 105°C from the top of the tower).

Subsequently, the thus-obtained granular base (100 parts by mass) was fed to a Lodige mixer (product of Matsuzaka Giken Co., Ltd., capacity: 20 L, equipped with a jacket). While the granular base was stirred by use of the main shaft (150 rpm), a mixture of a nonionic surfactant (20 parts by mass), sodium linear alkyl (C10-C13) benzenesulfonate (22 parts by mass), a fatty acid (C14-C18) sodium salt (4 parts by mass), polyethylene glycol (2 parts by mass), and water (4 parts by mass) was added to the mixer over three minutes. Thereafter, the resultant mixture was stirred for five minutes. Furthermore, crystalline sodium silicate (20 parts by mass) and zeolite (10 parts by mass) were added to the mixer for surface coating, to thereby yield a detergent base.

The detergent base (99 mass%) was mixed with example protease granules of the present invention (0.5 mass%) and a

perfume (0.5 mass%), to thereby produce an end product, granular detergent A.

(2) Raw materials employed

Nonionic surfactant: Emulgen 108KM (average mole number of ethylene oxide added: 8.5, product of Kao Corporation)

Aqueous solution of sodium polyacrylate: average molecular weight: 10,000 (produced by use of the method described in the examples of Japanese Patent Publication (*kokoku*) No. 2-24283)

Sodium carbonate: Dense ash (product of Central Glass Co., Ltd.)

Zeolite: Zeolite 4A (average particle size: 3.5 μm, product of Tosoh Corporation)

Polyethylene glycol: K-PEG6000 (average molecular weight: 8,500, product of Kao Corporation)

Crystalline sodium silicate: Powder SKS-6 (product of Hoechst Tokuyama)

Example protease granules of the present invention: granules prepared from each of purified samples of the example alkaline proteases of the present invention shown in Figs. 2 and 3 by the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990 (6 PU/g)

Fluorescent dye: Tinopal CBS-X (product of Ciba-Geigy Corp.)

Example 5

(1) Preparation of detergent

The slurry (solid content: 50 mass%) was spray-dried with hot air at 250°C, to thereby yield a granular base containing sodium polyacrylate (mass average molecular weight: 10,000) (7 mass%), sodium carbonate (26 mass%), sodium sulfate (20 mass%), sodium chloride (6 mass%), fluorescent dye (0.5 mass%), zeolite (40 mass%), and water (0.5 mass%).

Subsequently, the thus-obtained granular base (100 parts by mass) was fed to a Lodige mixer (product of Matsuzaka Giken Co., Ltd., capacity: 20 L, equipped with a jacket). While the granular base was stirred by means of the main shaft (150 rpm), a mixture of a nonionic surfactant (20 parts by mass), sodium linear alkyl (C10-C13) benzenesulfonate (22 parts by mass), a fatty acid (C14-C18) sodium salt (4 parts by mass), polyethylene glycol (2 parts by mass), and water (4 parts by mass) was added to the mixer over three minutes. Thereafter, the resultant mixture was stirred for five minutes. Furthermore, crystalline sodium silicate (20 parts by mass) and zeolite (10 parts by mass) were added to the mixer for surface coating, to thereby yield a detergent base.

The detergent base (95 mass%) was mixed with bleaching agent granules (2.8 mass%), bleaching activator granules (1.2 mass%), example protease granules of the present invention (0.5 mass%), and a perfume (0.5 mass%), to thereby produce an end product, granular detergent B.

(2) Raw materials employed

Nonionic surfactant: Emulgen 108KM (average mole number of ethylene oxide added: 8.5, product of Kao Corporation)

Aqueous solution of sodium polyacrylate: average molecular weight: 10,000 (produced by the method described in the examples of Japanese Patent Publication (*kokoku*) No. 2-24283)

Sodium carbonate: Dense ash (product of Central Glass Co., Ltd.)

Zeolite: Zeolite 4A (average particle size: 3.5 μm, product of Tosoh Corporation)

Polyethylene glycol: K-PEG6000 (average molecular weight: 8,500, product of Kao Corporation)

Crystalline sodium silicate: SKS-6 (product of Hoechst Tokuyama)

Example Protease granules of the present invention: granules prepared from each of the purified samples of the example alkaline proteases of the present invention shown in Figs. 2 and 3 by the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990 (6 PU/g)

Fluorescent dye: Tinopal CBS-X (product of Ciba-Geigy Corp.)

Bleaching agent granules: a sodium carbonate-hydrogen peroxide addition product (produced in a manner similar to that for producing bleaching agent granules described in paragraph [0019] of Japanese Patent Application Laid-Open (*kokai*) No. 2000-256699)

Bleaching activator granules: granules of sodium lauroyloxybenzenesulfonate (produced in a manner similar to that for producing bleaching activator granules described in paragraph [0018] of Japanese Patent Application Laid-Open (*kokai*) No. 2000-256699)

Example 6

Liquid detergent compositions (detergents C and D) shown in Table 2 were prepared.

Table 2

Components	Detergent C (mass%)	Detergent D (mass%)
Nonionic surfactant[1)]	25.0	-
Nonionic surfactant[2)]	5.0	-
Nonionic surfactant[3)]	10.0	-
Nonionic surfactant[4)]	-	9.0
Nonionic surfactant[5)]	-	9.0
Nonionic surfactant[6)]	-	2.5
Anionic surfactant[7)]	1.0	-
Silicone[8)]	-	0.8
Carboxylic acid-based polymer[9)]	2.0	-
Polymer[10)]	-	0.8
Citric acid	0.2	-
Calcium chloride	0.05	-
Monoethanolamine	4.0	-
Triethylene glycol phenyl ether	3.0	-
Propylene glycol	3.0	-
Ethanol	2.0	2.0
Sodium sulfite	0.2	-
Example Protease of the present invention[11)]	0.5	1.0
Perfume	0.5	0.5
Water	Balance	Balance
Total	100	100
Concentration upon use	20 g/30 L	40 g/30 L
pH of detergent solution	10.5	7.3

1) Polyoxyethylene (average mole number added: 7) alkyl ether having an alkyl group derived from a C12-C14 secondary alcohol (Softanol 70, product of Nippon Shokubai Kagaku Kogyo)

2) Polyoxyethylene (average mole number added: 12) alkyl ether having an alkyl group derived from a C12-C14 secondary alcohol (Softanol 120, product of Nippon Shokubai Kagaku Kogyo)

3) A product obtained by sequential block addition of EO (average mole number: 5), PO (average mole number: 2), and

EO (average mole number: 3) to a C10-C14 linear primary alcohol

4) Polyoxyethylene lauryl ether (average mole number of EO added: 8)

5) Polyoxyethylene lauryl ether (average mole number of EO added: 11.5)

6) Narrow range polyoxyethylene alkyl (sec-C_{12}/C_{13}) ether

7) Sodium linear alkyl (C10-C14) benzenesulfonate

8) Amide/ether-modified silicone polymer (BY16-906, product of Dow Corning Toray Silicone Co., Ltd.)

9) A phenoxypolyethylene glycol - acrylic acid - maleic acid copolymer synthesized by the method described in lines 6 through 13 of page 11 of Japanese Patent Application Laid-Open (*kokai*) No. 10-60476 (mass average molecular weight: 10,000, solid content: 51.2%)

10) A sodium salt of a pentene/maleic acid (ratio by mol: 50/50) copolymer (mass average molecular weight: 7,000)

11) A purified sample of each of the example alkaline proteases of the present invention shown in Figs. 2 and 3 (15 PU/mL)

Example 7

While sodium percarbonate and sodium carbonate (dense ash) of the components shown in Table 3 below were mixed while stirring, a 40% aqueous solution of sodium polyacrylate and sodium linear alkyl benzenesulfonate, or a nonionic surfactant, or sodium lauroyloxybenzenesulfonate were added to the mixture. Subsequently, to the resultant mixture were

added example protease granules of the present invention prepared by the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990, and the resultant mixture was stirred until a uniform mixture was obtained to give a bleaching agent.

Table 3

Components	Bleaching agent E (mass%)	Bleaching agent F (mass%)
Sodium percarbonate[1)]	72.0	72.0
Sodium carbonate (dense ash)	20.0	20.0
Anionic surfactant[2)]	2.0	-
Nonionic surfactant[3)]	-	2.0
Sodium polyacrylate[4)]	1.0	1.0
Sodium lauroyloxybenzenesulfonate	4.0	4.0
Example Protease of the present invention[5)]	1.0	1.0

1) Particle size: 500 to 700 μm

2) Sodium linear alkyl (C12-C14) benzenesulfonate

3) Polyoxyethylene alkyl ether (number of carbon atoms of the alkyl group: 12 to 14, average mole number of EO added: 12)

4) Average molecular weight: 8,000

5) Granules (6 PU/g) prepared from each of purified samples of the alkaline proteases of embodiments of the present invention shown in Figs. 2 and 3 by the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990

Example 8

Detergent compositions for an automatic dishwasher (detergents G and H) shown in Table 4 below were prepared.

Table 4

Components	Detergent G (mass%)	Detergent H (mass%)
Pluronic L-61[1)]	-	4.0
Softanol EP-7085[2)]	4.0	-
Trisodium citrate	-	30.0
Sodium tripolyphosphate	30.0	-
Sodium percarbonate	20.0	20.0
Sodium carbonate	20.0	20.0
Amorphous silicate[3)]	10.0	10.0
AA-MA[4)]	4.0	4.0
Sodium sulfate	10.0	10.0
α-Amylase[5)]	1.0	1.0
Example Protease of the present invention[6)]	1.0	1.0

1) Polyoxyethylene - polyoxypropylene copolymer (average molecular weight: 2,000)

2) A product obtained by adding to a C12-C14 sec-alcohol ethylene oxide (7 mol) and propylene oxide (8.5 mol)

3) JIS No. 2 sodium silicate

4) An acrylic acid - maleic acid copolymer

5) Duramyl 60T (registered trademark; product of Novozymes)

6) Granules (6 PU/g) prepared from each of purified samples of the alkaline proteases of the embodiments of the present invention shown in Figs. 2 and 3 by the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990

Example 9

A detergent composition for hard surfaces (detergent J) was prepared from components shown in Table 5 below.

Table 5

Components	Detergent J (mass%)
Anionic surfactant[1)]	15.0
Nonionic surfactant[2)]	5.0
Nonionic surfactant[3)]	5.0
Amphoteric surfactant[4)]	7.5
Amphoteric surfactant[5)]	4.0
Citric acid	1.0
Polypropylene glycol[6)]	2.0
Ethanol	5.0
Example Protease of the present invention[7)]	1.0
Perfume, water, etc./pH modifier	54.5
Total	100.0

1) Sodium polyoxyethylene (EOP = 4) alkyl (C12) ether sulfate

2) Polyoxyethylene (EOP = 8) alkyl (C12) ether

3) Alkyl (C12) polyglucoside (condensation degree: 1.3)

4) Mono long-chain tertiary alkyl (C12) dimethylamine oxide

5) Alkyl (C12) hydroxydimethyl sulfobetaine

6) Molecular weight: 10,000

7) Each of the purified samples of the example alkaline protease of the present invention shown in Figs. 2 and 3 (15 PU/mL)

Example 10

Granular detergents shown in Table 6 below were prepared by use of the aforementioned detergent A (see Example 2).

Table 6

Components (mass%)	Detergent K	Detergent L	Detergent M	Detergent N
Detergent base of Example 2	98.4	98.3	98.5	97.2
Perfume	0.5	0.5	0.5	0.5
Example Protease of the present invention[1)]	0.5	0.5	0.5	0.5
Conventional protease[2)]	0.6			0.6
Cellulase[3)]		0.7		0.7
Lipase[4)]			0.5	0.5

1) Granules (6 PU/g) prepared from each of purified samples of the example alkaline proteases of the present invention shown in Table 1 by the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990

2) Protease K-16 described in Japanese Patent Application Laid-Open (*kokai*) No. 5-25492, the activity thereof having been regulated to 5 PU/g by the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990

3) KAC-500 (registered trademark; product of Kao Corporation)

4) Lipolase 100T (registered trademark; product of Novozymes)

The present invention enables production of an alkaline protease which has for example, a high thermal stability, exhibits activity even in the presence of a fatty acid at a high concentration and excellent detergency against complex